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EVALUATION OF METHYLATION PROFILES OF AN EPIDERMAL GROWTH FACTOR RECEPTOR GENE IN A HEAD AND NECK SQUAMOUS CELL CARCINOMA PATIENT GROUP

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ABSTRACT

Upregulation of the epidermal growth factor receptor (*EGFR*) gene has shown an important impact on the development of head and neck cancers due to its important regulation role on multiple cell signaling pathways. The aim of this study was to investigate the methylation pattern of the promoter region of the *EGFR* gene between head and neck squamous cell carcinoma (HNSCC) patients and a control group. Forty-seven unrelated HNSCC patients, clinically diagnosed at the Department of Otorhinolaryngology, Dışkapı Yıldırım Beyazıt Training and Research Hospital, Ankara, Turkey, and 48 unrelated healthy volunteers from different geographic regions of Turkey, were included in this study. Methylation status of the promoter region of the *EGFR* gene was detected by methylation-specific-polymerase chain reaction (MS-PCR). The correlation between *EGFR* gene promoter methylation profiles and clinical characteristics were examined using the χ^2 test. Methylation was observed in 79.0% of HNSCC patients, whereas this ratio was 90.0% in healthy individuals. The results show that promoter region methylation of the *EGFR* gene was not associated with HNSCC development in the studied Turkish patient group. In addition, the methylation status of the *EGFR* gene promoter was not found to be related to age, gender or tumor stage.

Keywords: Epidermal growth factor receptor (*EFGR*) gene; epigenetics; head and neck squamous cell carcinoma (HNSCC); methylation; methylation-specific-polymerase chain reaction (MS-PCR).

INTRODUCTION

Head and neck cancers are defined as a group of malignant diseases originating from the larynx, pharynx and oral cavity. They have a high incidence, being classified as the sixth most common cancer worldwide. On the other hand, if they are not diagnosed at an early stage, they are associated with high mortality rate [1]. The most frequently seen histological type of head and neck cancers is the squamous cell carcinoma (SCC), corresponding to approximately 90.0% of cases [2]. Tobacco and alcohol consumption, viral infections such as Epstein-Barr Virus and Human Papilloma Virus (16/18), deficiencies of some vitamins and micronutrients, are considered as promoting factors of this tumor type [3-5].

Various mutations, polymorphisms in oncogenes and also epigenetic changes are responsible for development and progression of head and neck squamous cell carcinoma (HNSCC) [6]. In addition to these alterations, chromosomal inversions, deletions, translocations, gains, losses and trisomy of chromosome 7 are common in HNSCC [7-9]. Differential gene expression patterns due to mutations in several genes including tumor protein 53 (*TP53*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), FAT atypical cadherin 1 (*FAT1*), phosphatase and tensin homolog (*PTEN*), HRas proto-oncogene, GTPase (*HRAS*), phos-phatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*) and epidermal growth factor receptor (*EGFR*) gene had been implicated in HNSCC [10,11].

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Among these alterations, upregulation of the *EGFR* gene has an important impact in the development of head and neck cancers [12]. This receptor tyrosine kinase belongs to ErbB family of cell surface receptors and is associated with carcinogenesis due to its important regulation role on multiple cell signaling pathways [12]. The phos-phorylated receptor can activate mitogen-activated protein kinase (MAPK), protein kinase B (Akt), extracellular signal-regulated kinase (Erk), janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways. The activation of these pathways result in cell proliferation, apoptosis, angiogenesis, invasion and metastasis [13]. Upregulation of the EGFR protein was seen in approximately 90.0% HNSCC, however, amplification of this gene locus was not prevalent being seen only in 10.0-30.0% of cases [14]. This suggests that other mechanisms rather than gene amplification may be associated with *EGFR* overexpression.

In addition to nucleotide changes in the genome, epigenetic alterations are the other group of events that cause carcinogenesis. DNA and protein methylation profiles are one of the most consistent epigenetic changes in human cancers [15]. Cancer cells generally show a drastic change in DNA methylation status, either exhibiting DNA hypomethylation, which causes overexpression of oncogenes or accompanying region-specific hypermethylation that results in silencing of tumor suppressor genes [16-18]. Identification of DNA methylation patterns of specific gene promoter regions can be used as biomarkers for early diagnosis, classification, prognosis and therapy of human cancers including HNSCC [19,20]. Tumor suppressor *CDKN2A/p16*, cadherin 1 (*CDH1*), death associated protein kinase 1 (*DAPK*) and O-6-methylguanine-DNA methyltransferase (*MGMT*) genes were described as hypermethylated in the larynx carcinoma, whereas the WNT signaling pathway regulator (APC) and ubiquitin C-terminal hydrolase L1 (UCHL1) were reported as hypermethylated in nasopharyngeal carcinoma [21]. Hypermethylation in the promoter region of semaphorin 3B (*SEMA3B*) was shown in oral squamous cell carcinoma tissues [22]. On the other hand, in HNSCC, hypermethylation correlates with the stage of the disease and its potential to metastasis [23]. For example, aberrant methylation of *DAPK*, netrin 1 receptor (*DCC*) and *MINT31* genes correlate with advanced stages of the disease and metastasis [24,25].

The relationship between EGFR protein methylation and HNSCC was shown in several studies. Saloura *et al.* [26] stated that histone-lysine N-methyltransferase NSD3 (*WHSC1L1*) mediated methylation of EGFR protein resulted in enhanced cell cycle progression *via* increasing *EGFR* interaction with proliferating cell nuclear antigen (*PCNA*) in HNSCC cells. In another study, the correlation between the expression level of methyl-EGFR and protein arginine N-methyltransferase 1 (*PRMT1*) was shown in patients with head and neck cancer [27].

Although there are studies that show the association between *EGFR* gene overexpression and EGFR protein methylation status in HNSCC development, they are not focused on *EGFR* gene promoter region methylation. The reversibility of epigenetic changes by either reactivation or suppression of epigenetically suppressed or activated genes, is thought to be important for the development of new treatment strategies in cancer treatment [28]. From this point of view, in this study, we aimed to investigate the methylation pattern of the promoter region of the *EGFR* gene between Turkish HNSCC cancer patients and a control group for the first time in order to identify the contribution of this difference to the development and progression of the disease.

MATERIALS AND METHODS

Study Population. Forty-seven unrelated Turkish HNSCC patients who were clinically diagnosed at the Department of Otorhinolaryngology, Dışkapı Yıldırım Beyazıt Training and Research Hospital, Ankara, Turkey, and 48 unrelated healthy volunteers from different geographic regions of Turkey, were included in this study. The control group was selected to match the patients in terms of demographic data including age and gender. All individuals in the study groups gave informed consent and approval of the local ethics committee was obtained from Dışkapı Yıldırım Beyazıt Training and Research Hospital [2018.10.15; #55/18]. The study was conducted in accordance with the guidelines of the Declaration of Helsinki.

Clinicopathological parameters of patients were determined by both tumor type and tumor stage. Tumor stage 1 (T1) represents the primary tumor (<2 cm) and at this stage no cancer cells are present in nearby structures such as lymph nodes or distant sites. Tumor stage 2 (T2) shows the tumors that measure between 2-4 cm with no cancer cells in nearby structures, lymph nodes or distant sites. Tumor stage 3 (T3) shows either tumors larger than 4 cm across with no cancer cells present in nearby structures, lymph nodes or distant sites, or any size but with cancer cells that present in one lymph node that is located on the same side of the head or neck as the primary tumor. Finally, tumor stage 4 (T4) represents the head and neck cancer tumor in any size but is spreading to nearby structures, lymph nodes, invaded deeper tissues or distant sites (Table 1).

DNA Isolation and Bisulfite DNA Modification. Genomic DNA was isolated from both HNSCC patient and control group's peripheral blood samples using QIAamp®

Table 1. Clinicopathological parameters of the control and patient groups.

	Control Group (*n*=48) (%)	Patient Group (*n*=47) (%)
Gender	males: 36 (75.00); females: 12 (25.00)	males: 41 (87.00); females: 6 (13.00)
Median age	58	59
Tumor type	–	Larynx: 31 (65.96) Hypopharynx: 5 (10.64) Lip: 3 (6.39) Tongue: 2 (4.26) Buccal: 2 (4.26) Auricular: 1 (2.13) Retromolar/oral: 1 (2.13) Paranasal sinus: 1 (2.13) Parotid gland: 1 (2.13)
Tumor stage	–	T1: 10 (21.27) T2: 10 (21.27) T3: 20 (42.55) T4: 7 (14.89)

DNA Blood Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Bisulfite DNA modification was performed using EZ DNA Methylation Gold™ Kit (ZymoResearch, Irvine, CA, USA). Bisulfite modified DNAs were stored at –80 °C until they were used for methylation-specific-polymerase chain reaction (MS-PCR) analyses.

Methylation-Specific-Polymerase Chain Reaction. Promoter methylation of *EGFR* gene was detected by MS-PCR. Bisulfite modified DNAs were used as the template for this analysis. As a positive methylation control, bisulfite converted universal methylated human DNA standard (ZymoResearch) was used. Specific primers for both methylated and unmethylated DNA sequences were obtained for the *EGFR* gene (Table 2) [29]. Two sets of primers were specific for nucleotides –130 to –300 in the 5' untranslated region (5'UTR) of the human EGFR promoter. The MS-PCR mixture contained 10 × LightCycler®480 SYBR Green I Master Mix (Roche Diagnostics International AG, Rotkreuz, Switzerland), 10 × PCR primers and Bisulfite-modified DNA (500 ng) in a final volume of 20 µL. Control DNA was used for each set of PCR. The MS-PCR reaction was performed using the LightCycler®480 Instrument (Roche Diagnostics International AG). The MS-PCR conditions were as follows: pre incubation at 95 °C for 5 min., amplification for 45 cycles at 95 °C for 10 seconds, 60 °C for 10 seconds and 72 °C for 10 seconds with single acquisition mode at 72 °C. Melting curve analysis was performed at 95 °C for 5 seconds, 65 °C for 1 min. and continuous acquisition mode at 97 °C.

At the end of the methylated and unmethylated MS-PCR reactions, cycle threshold (Ct) values were evaluated. In general, Ct values smaller than 35 represents the true positive cases. Thus, Ct values that were equal to or lower than 35 were considered to be positive. The values between 35-40 can be false positive so we did not accept Ct values over 35 as positive.

Statistical Analyses. All statistical analyses were performed using the Statistical Package for Social Science (SPSS), version 21.0 (IBM Inc., Armonk, NY, USA). The correlation between EGFR promoter methylation profile and clinical characteristics were examined using the χ^2 test. A two-sided test was considered statistically to be significant at $p < 0.05$.

RESULTS

Clinicopathological Parameters of Control and Patient Groups. Control group were selected to match the patients in terms of demographic data including age and gender. The median ages of control and patient groups were 58 and 59, respectively. On the other hand, 13.0% of the patients were female and 87.0% were male, whereas 25.0% of the healthy individuals were female and 75.0% were male. The most common tumor originated on the larynx (65.96%), followed by hypopharynx (10.64%) among the HNSCC patient group in our study. According to pathological staging, 21.27% patients were reported as being in T1, 21.27% in T2, 42.55% in T3 and 14.89% in T4 stages (Table 1).

Methylation Profile. Promoter methylation of the *EGFR* gene was detected by MS-PCR following bisulfite modification of DNAs (Figure 1 and Figure 2). Cycle threshold values obtained from both healthy individuals and patients were compared with the Ct values of control DNA, and the methylation profiles were determined for each individual. If the methylated reaction is positive and the unmethylated reaction is negative or both reactions are

Table 2. Polymerase chain reaction primers for methylated and unmethylated DNA sequences for the *EGFR* gene.

Primer Name	Sequences (5'>3')	PCR Product
EGFR-M Forward EGFR-M Reverse	TGT TTT TTT CGC GTT TCG GTT CGC GC CGT CTA AAC GAC GAC GAC CGC CG	150 bp
EGFR-UM Forward EGFR-UM Reverse	TGT TTT GTT TTT TTG TGT TTT GGT TTG TGT CAT CCA ATC TAA ACA ACA ACA ACC ACC A	150 bp

M: methylated; UM: unmethylated.

Table 3. Determination of methylation profile after methylation-specific-polymerase chain reaction.

MS-PCR		
Methylated	**Unmethylated**	**Methylation Profile**
[–]	[+]	unmethylated
[+]	[–]	methylated
[+]	[+]	methylated
[–]	[–]	excluded from study

positive, the methylation status is evaluated as methylated. On the other hand, if the methylated reaction is negative and the unmethylated reaction is positive, methylation status is determined as unmethylated. Samples with negative reaction of both methylated and unmethylated were excluded from the study (Table 3 and Table 4).

According to the MS-PCR results, methylation was observed in 79.0% of patients, whereas methylation was not observed in 21.0% of patients. On the other hand, in

Table 4. Methylation profiles of control and patient groups.

Control Group	MS-PCR				Methylation Profile	Patient Group	MS-PCR				Methylation Profile
	Ct	M	Ct	UM			Ct	M	Ct	UM	
Positive Control[a]	27.33	[+]	40.00	[–]	M	Positive Control[a]	27.33	[+]	40.00	[–]	M
C-1	34.37	[+]	32.28	[+]	M	P-1	33.72	[+]	31.41	[+]	M
C-2	32.86	[+]	30.67	[+]	M	P-2	29.87	[+]	29.77	[+]	M
C-3	31.43	[+]	28.88	[+]	M	P-3	33.21	[+]	31.95	[+]	M
C-4	32.92	[+]	29.35	[+]	M	P-4	31.68	[+]	29.50	[+]	M
C-5	31.82	[+]	29.74	[+]	M	P-5	32.60	[+]	29.89	[+]	M
C-6	31.93	[+]	28.99	[+]	M	P-6	30.82	[+]	29.36	[+]	M
C-7	31.91	[+]	28.57	[+]	M	P-7	32.47	[+]	29.00	[+]	M
C-8	35.95	[–]	32.27	[+]	UM	P-8	31.66	[+]	28.34	[+]	M
C-9	31.25	[+]	29.97	[+]	M	P-9	30.84	[+]	29.53	[+]	M
C-10	29.84	[+]	27.52	[+]	M	P-10	32.65	[+]	29.36	[+]	M
C-11	32.69	[+]	30.40	[+]	M	P-11	35.32	[–]	33.99	[+]	UM
C-12	33.58	[+]	33.50	[+]	M	P-12	36.56	[–]	34.23	[+]	UM
C-13	33.67	[+]	29.72	[+]	M	P-13	32.74	[+]	30.43	[+]	M
C-14	34.55	[+]	30.58	[+]	M	P-14	34.57	[+]	32.49	[+]	M
C-15	33.94	[+]	30.88	[+]	M	P-15	33.73	[+]	30.99	[+]	M
C-16	33.88	[+]	29.60	[+]	M	P-16	35.42	[–]	33.56	[+]	UM
C-17	34.66	[+]	32.72	[+]	M	P-17	35.56	[–]	31.58	[+]	UM
C-18	32.31	[+]	29.43	[+]	M	P-18	34.46	[+]	31.25	[+]	M
C-19	33.84	[+]	30.86	[+]	M	P-19	36.38	[–]	33.45	[+]	UM
C-20	33.76	[+]	30.72	[+]	M	P-20	35.12	[–]	32.46	[+]	UM
C-21	33.30	[+]	29.73	[+]	M	P-21	35.98	[–]	33.72	[+]	UM
C-22	34.56	[+]	29.86	[+]	M	P-22	33.67	[+]	31.98	[+]	M
C-23	33.40	[+]	30.30	[+]	M	P-23	34.28	[+]	31.94	[+]	M
C-24	33.97	[+]	31.45	[+]	M	P-24	34.77	[+]	31.80	[+]	M
C-25	33.30	[+]	30.37	[+]	M	P-25	33.33	[+]	31.58	[+]	M
C-26	32.88	[+]	30.47	[+]	M	P-26	32.55	[+]	30.69	[+]	M
C-27	32.35	[+]	31.44	[+]	M	P-27	33.65	[+]	31.66	[+]	M
C-28	34.17	[+]	31.74	[+]	M	P-28	32.99	[+]	29.57	[+]	M
C-29	33.24	[+]	30.55	[+]	M	P-29	33.22	[+]	28.42	[+]	M
C-30	32.74	[+]	28.52	[+]	M	P-30	35.51	[–]	28.80	[+]	UM

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C-31	33.21	[+]	27.67	[+]	M	P-31	33.48	[+]	28.53	[+]	M
C-32	35.24	[+]	30.34	[+]	UM	P-32	34.27	[+]	29.92	[+]	M
C-33	32.86	[–]	27.26	[+]	M	P-33	32.63	[+]	27.98	[+]	M
C-34	32.42	[+]	28.35	[+]	M	P-34	32.88	[+]	27.72	[+]	M
C-35	32.29	[+]	28.39	[+]	M	P-35	33.68	[+]	29.86	[+]	M
C-36	33.80	[+]	28.59	[+]	M	P-36	33.67	[+]	28.82	[+]	M
C-37	34.38	[+]	27.65	[+]	M	P-37	34.92	[+]	30.58	[+]	M
C-38	33.61	[+]	27.66	[+]	M	P-38	33.78	[+]	29.73	[+]	M
C-39	33.81	[+]	28.85	[+]	M	P-39	35.75	[–]	28.32	[+]	UM
C-40	32.49	[+]	28.40	[+]	M	P-40	34.72	[+]	29.49	[+]	M
C-41	33.84	[+]	29.66	[+]	M	P-41	32.83	[+]	28.61	[+]	M
C-42	34.29	[+]	28.94	[+]	M	P-42	35.31	[–]	30.58	[+]	UM
C-43	33.97	[+]	29.58	[+]	M	P-43	32.45	[+]	27.63	[+]	M
C-44	35.82	[–]	30.24	[+]	UM	P-44	33.76	[+]	29.67	[+]	M
C-45	35.75	[–]	30.19	[+]	UM	P-45	32.99	[+]	28.25	[+]	M
C-46	35.90	[–]	30.22	[+]	UM	P-46	33.71	[+]	28.53	[+]	M
C-47	33.00	[+]	28.23	[+]	M	P-47	32.89	[+]	28.45	[+]	M
C-48	33.59	[+]	29.34	[+]	M						

MS-PCR: methylation-specific-polymerase chain reaction; Ct: cycle threshold; M: methylated; UM: unmethylated.
[a] Positive methylation control [bisulfite converted universal methylated human DNA standard (ZymoResearch)]; Ct value is 27.46.

Table 5. The *EGFR* gene promoter methylation profile.

Sample	EGFR Promoter Methylation Profile (%)		*p* Value
	Methylated	Unmethylated	
Control	43 (90.0);	5 (10.0)	0.121[a]
Patient	37 (79.0)	10 (21.0)	

[a] $p > 0.05$ (Pearson χ^2 analyses).

the control group, methylation was observed in 90.0% healthy individuals (Table 5).

The methylation profile of the *EGFR* gene promoter was also compared with age, gender, and clinicopathological characteristics in the control and patient groups (Table 6). There is no statistically significant difference in terms of age, gender and clinicopathological characteristics.

Table 6. Relation of the *EGFR* gene promoter methylation profile of patient group with age, gender and clinicopathological parameters.

Parameters	*n*	EGFR Methylation Profile		*p* Value
		Methylated	Unmethylated	
Age:				
<50	4	3	1	0.908[a]
>50	43	34	9	
Gender:				
males	41	33	8	0.494[a]
females	6	4	2	
Tumor stage:				
I-II	20	14	6	0.184[a]
III-IV	27	23	4	

[a] $p > 0.05$ (Pearson χ^2 analyses).

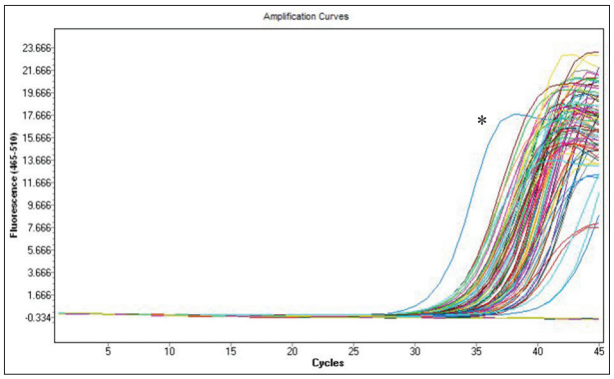

Figure 1. Amplification curves of methylated reactions from both HNSCC and healthy control groups.
* Positive methylation control.

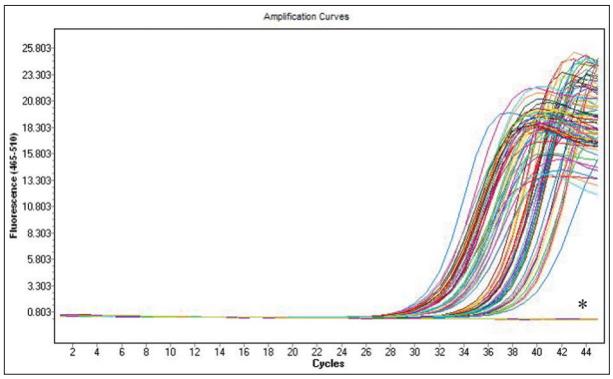

Figure 2. Amplification curves of unmethylated reactions from both HNSCC and healthy control groups.
* Positive methylation control.

DISCUSSION

The average 5-year survival in HNSCC patients is approximately 60.0% and this ratio lowers with increasing stages at diagnosis. This indicates a need for noninvasive tests that facilitate the detection of early disease [30]. DNA methylation is an early event in tumorigenesis of HNSCC, thus, identification of methylation profiles of specific genes can be used as biomarkers and provide great promise for early detection and treatment in HNSCC [31]. Differences in DNA methylation profiles in the CpG islands help us to understand the pathogenesis of complex diseases including cancer [32]. Methylation profiles not only show the differentiation of normal cells to cancerous cells, but also help to define specific cancer types [33]. In addition, epigenetic studies provide margin assessment that can be helpful to surgeons and doctors in clinics. In selected patients with HNSCC requiring comprehensive resection, rapid molecular margin analysis using MS-PCR, is feasible and may be performed intraoperatively [34]. (Author: this sentence is not complete) PAGE Frequent hypermethylation of tumor-related genes were observed in oral squamous cell carcinoma (OSCC) and HNSCC [35]. The genes found to be inactivated by DNA methylation events are involved in the cell cycle control, apoptosis, WNT signaling pathway and DNA repair mechanisms [35]. A systematic search in databases for HNSCC resulted in some reported genes that show hypermethylation on their promoter region (*ALDH3A1*, *CCNA1*, *CDH1*, *CDKN2A*/*p16*, *CDKN2B*/*p15*, *DAPK*, *DCC*, *EDNRB*, *ERCC1*, *ESR1*, *FANCC*, *FHIT*, *GALR1*, *GALR2*, *HIC1*, *HOTAIR*, KIF1A, *LKB1*, *MGMT*, *MLH1*, *PTCH1*, *RAR*β*2*, *RASSF4*, *RASSF5*, *RUNX3*, *SEMA3B*, *SPARC*, *TAP1*, *TCF21*, *TIMP3* and *TRG*) [36]. However, some of these potential epigenetic biomarkers have not yet been clinically implemented [37].

In addition to these genes, *EGFR* had also been implicated in HNSCC. Upregulation of the *EGFR* gene was shown to be important in head and neck cancers due to its important regulation role on multiple cell signaling pathways [12]. The *EGFR* gene expression was seen both in premalignant oral lesions and invasive HNSCC tumor samples being unrelated to tumor stage [38]. Overproduction of EGFR protein was commonly seen (approximately 90.0%) in HNSCC patients, whereas amplification of this gene locus was not frequent (10.0-30.0%) [14]. In some of the studies, the relationship between methylated EGFR protein and HNSCC was shown [26,27]. However, none of these studies focused on if the upregulation of the *EGFR* gene can be a result of *EGFR* gene promoter region methylation in HNSCC patients and can be a prediction marker for the disease by comparing healthy individuals.

In our study, we aimed to consider the mechanisms of overexpression of the *EGFR* gene in Turkish HNSCC patient group rather than gene amplification. Thus, we focused on methylation status of the *EGFR* gene promoter region and wanted to contribute to the gene lists in the databases. On the other hand, it is vital to determine non-invasive tests from blood samples that facilitate the detection of early disease. Studying DNA methylation profiles from peripheral blood samples is an easy and noninvasive way. There have been several recent reports on blood-based methylation biomarkers for various solid tumor types including HNSCC [30,39-42]. DNA methylation is amenable to measure and readily available in peripheral blood. The results show most likely *EGFR* methylation of white blood cells such as might be observed in a specific immune response to the tumor and of circulating tumor cells, if any. To the best of our knowledge, the *EGFR* gene promoter methylation profile for Turkish HNSCC patients has so far not been studied in peripheral blood samples and compared with a healthy control group, so the results of this study may contribute to the literature.

According to clinicopathological data of the patients; the methylation status of the *EGFR* gene promoter was not found to be related to age, gender or tumor stage. Although some publications in the literature suggest that different methylation profiles vary, depending on age and gender for various genes in different types of cancer and normal tissues [43-45], no such difference was found to be statistically significant for the *EGFR* gene in our HNSCC patient group. In addition, methylation of the *EGFR* gene in the promoter region was not associated with HNSCC development when compared with the control group.

To the best of our knowledge, there are no studies in the literature showing *EGFR* gene promoter methylation status from blood samples of HNSCC patients and comparing them with healthy individuals for the prediction of early disease. The methylation status of EGFR CpG islands was examined in a series of solid tumor types including head and neck in the USA, and EGFR hypermethylation was detected only in 35.0% of cases and not considered statistically significant [46]. On the other hand, in our study, 79.0% of Turkish HNSCC patients were found to be methylated. Although in this study Montero *et al.* [46] used pyrosqeuencing results of tumor samples, it is important to show the possible relation between EGFR promoter methylation status and HNSCC from other ethnic groups.

In conclusion, promoter methylation of specific genes is emerging as one of the most promising cancer detection strategies and to be a tumor-specific marker for early diagnosis of HNSCC. The results of this study suggest that the *EGFR* gene promoter methylation status is not associated with HNSCC in the studied Turkish patient group. However, in order to make a more definitive conclusion, it is necessary to increase the number of cases and other target genes that have important roles in the progression of HNSCC included in our future studies.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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